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THERAPY FOR PRIMARY AND METASTATIC CANCERS

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THERAPY FOR PRIMARY AND METASTATIC CANCERS

BACKGROUND

[0001] This application claims priority to U.S. Provisional Patent Application, Serial Number 60/443,095, entitled "Treatment for Metastatic Cancer," filed on January 28, 2003, the entire content of which is hereby incorporated by reference.

[0002] One aspect of the present invention relates to an immunotherapy for the treatment of metastatic tumors. The immunotherapeutic agents and methods of the invention relate to an administration of a physiological stress (e.g. heat) and a genetically engineered oncolytic virus directed either simultaneously or sequentially, to a treatment area, which results in subsequent tumor regression both locally and distally.

[0003] Cancer can be defined as a malignant neoplasm anywhere in the body of a person or animal. Cancer that spreads locally, or to distant parts of the body is called a metastasis. An example of the metastasis is a transfer of cells from a malignant tumor by way of the bloodstream or lymphatic fluid. There are various cancers that are characterized by the uncontrolled growth of cells that disrupt body tissue or metabolism (e.g. liver cancer, breast cancer, leukemia, etc.), wherein the proliferation destroys the adjacent tissues and finally causes death of the body by a physical block of the vessels and organs (Hanahan and Weinberg (2000). The hallmark of cancer. *Cell* 100.57-70). Thus, the two major characteristics of cancer cells are their immortality and their ability to form a metastasis.

[0004] **I. Available treatments for cancer.** Although cancer has been known for thousands of years, only recently has modern technical expertise allowed for possible treatments of cancer. Furthermore, the mechanism of action for these diverse diseases are becoming understood to the point where direct molecular intervention is possible. At present, the clinically available treatments for cancer are surgery, radiotherapy, hyperthermic therapy, chemotherapy, gene therapy, immunotherapy, and others.

[0005] **Surgery.** Currently, the most effective treatment of cancer still is surgery in combination with radiotherapy, chemotherapy, immunotherapy, hyperthermic therapy, etc. When cancer is diagnosed early, the 5-year survival rate after surgical treatment can be as high as 80% for various types of cancer patients. Unfortunately, in most cases the disease has

already developed into late stages (stages III or IV) when patients were diagnosed. Late stage cancer cells typically have already migrated through blood or lymph vessels to distant locations throughout the body, and surgical treatment is neither practical nor effective in controlling the disease. Another drawback of a surgical treatment is that surgery cannot be applied to widespread measle-like-metastatic cancer. A further drawback to surgical treatment is the physical complications and increased risk of cancer metastasis in the patient following surgery.

[0006] **Radiotherapy:** Radiation therapy is a treatment used to shrink or destroy solitary cancers that cannot be safely or completely removed by surgery. It is also used to treat cancers that are not affected by chemotherapy. Radiotherapy utilizes radiation at levels thousands of times higher than the amount used to produce a chest x-ray. This intense radiation destroys the ability of cells to divide and to grow. Both normal and cancer cells are affected, but the radiation treatment is designed to maximize tumor killing effect and minimize normal tissue killing effect. Maximizing the tumor killing effect is one reason radiation therapy is given in a series of treatments rather than one treatment. In addition to cancer cells, some normal cells will also be killed by the radiation. Some side effects may be apparent because of these normal cells being killed. Usually these side effects are temporary and outweighed by the benefits of killing cancer cells. However, it is noteworthy that radiotherapy only kills cancer cells in the region that has been radiated, but does not affect cancer cells distant from the radiated region. Moreover, some specific biological features of cancer cells (e.g., resistance to radiation, size of a tumor, the proportion of anoxia cells in the cancer), may make particular cancers less susceptible to radiotherapy.

[0007] **Hyperthermic therapy:** Hyperthermia therapy or heat therapy, raises the temperature of whole body or a local region by various means known in the art. The hyperthermic techniques to elevate the temperature of a local region are primarily radiations in different energy range (e.g., ultrasound, microwave, radiofrequency, etc.). Although the mechanism of hyperthermia therapy for the treatment of cancer is not fully understood, hyperthermia alone or in combination with other treatments such as radiotherapy and chemotherapy have been demonstrated to have an anti-cancer effect (Falk and Issels (2001) Hyperthermia in oncology. *Int. J. Hyperthermia* 17:1-18). Although not wanting to be bound by theory, hyperthermia changes the microenvironment of cancer cells, and leads to denaturalization and necrosis/apoptosis. Currently, there are still difficulties to optimize the

conditions of hyperthermia. For example, hyperthermic treatment is difficult for deep-seated malignant tumors, and the measurement of the actual temperature distribution in the tumor and in the immediately adjacent tissues can be inconsistent. Moreover, prior art does not demonstrate that hyperthermia is effective to treat cancer distant from the site where heat is applied.

[0008] Chemotherapy: Chemotherapy is the use of an anti-cancer (cytotoxic) drug to destroy cancer cells. Currently, there are over 50 different chemotherapy drugs available. Although some chemotherapy drugs are given alone, often several drugs may be combined (i.e. combination chemotherapy). The type of specific treatment depends on many things, including the type of cancer, and how far it has spread from the origin. Chemotherapy kills fast-dividing cancer cells as well as fast-dividing normal cells such as blood cells, skin cells and gastrointestinal cells. Therefore, the application of chemical drugs to treat cancer is accompanied by severe side effects. It is also found that chemotherapy is not very effective to treat metastatic cancer. The apoptosis-resistant cancer cells are not susceptible to chemical drugs even at high doses since the mechanism for most chemical drugs is to induce the apoptosis of cancer cells.

[0009] Gene therapy: Gene therapy has developed rapidly as a new type of treatment for cancer. There are many types of vectors to deliver therapeutic genes specifically targeting cancer cells. These vectors include adenovirus vectors, adeno-associated viruses, and liposomes (Anderson (1998) Human gene therapy. Nature 392:25-30). However, various kinds of side effects and low delivering efficiency of these vectors have not yet been conquered. Hence, the clinical application of gene therapy is limited. The concept of using an oncolytic virus to treat cancer was unveiled a decade ago (Barker and Berk (1987) Adenovirus proteins from E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156:107-21). The clinical application of oncolytic virus has made great progress ever since, and approximately a dozen of different oncolytic viruses have entered clinical trials (Kirn et al. (2001). Replication-selective virus therapy for cancer: Biological principle, risk management and future directions. *Nature* 7:781-787). Among these oncolytic viruses, adenovirus *dl*1520 has been best studied. In contrast to wild-type adenovirus, *dl*1520 is a variant adenovirus where a fragment of 827 bp in E1b region is deleted so that *dl*1520 does not express E1b-55kDa protein. The variant adenovirus *dl*1520 does not replicate in normal cells, but selectively replicate in cancer cells where the

tumor-suppressor gene *p*53 is dysfunctional and eventually lyse cancer cells. The clinical trials have demonstrated that (1) oncolytic virus is safe to patients and environment; (2) the efficacy of variant adenovirus *dl*1520 to suppress cancer growth is not as good as expected; (3) the combined treatment of oncolytic virus *dl*1520 with chemical anti-cancer drugs is effective to treat cancer to some extent (Ries and Kirn (2002) ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus. *British Journal of cancer* 86:5-11). Methods and compositions for treating neoplastic conditions by viral based therapy were disclosed in U.S. Patent 5,677,178 ("the McCormick '178 Patent"), titled "Cytopathic Viruses for Therapy and Prophylaxis of Neplasia," which issued on October 14, 1997 having McCormick et al., listed as inventors. In the McCormick '178 Patent, Methods and compositions for treating neoplastic conditions by viral-based therapy are provided. A mutant virus lacking viral proteins which bind and/or inactivate p53 or RB are administered to a patient having a neoplasm which comprises cells lacking p53 and/or RB function. The mutant virus is able to substantially produce a replication phenotype in neoplastic cells but is substantially unable to produce a replication phenotype in non-replicating, non-neoplastic cells having essentially normal p53 and/or RB function. The preferential generation of replication phenotype in neoplastic cells results in a preferential killing of the neoplastic cells, either directly or by expression of a cytotoxic gene in cells expressing a viral replication phenotype. However, there have been no prior art reports to demonstrate that a genetically engineered oncolytic viruses are effective to treat tumors distant from the site where viruses are administrated.

[0010] Immunotherapy: Approximately 90% of cancer patients die from metastasis, and there is virtually no effective treatments for cancer metastasis. Immunotherapy classically is a process by which an allergy patient is exposed to gradually increasing amounts of an allergen for the purpose of decreasing sensitivity to the allergen. The concept of immunotherapy for cancer treatment is based upon similar research that revealed that the immune system plays a central role in protecting the body against cancer and in combating cancer that has already developed. Although this latter role is not well understood, there is amble evidence that supports the role of the immune system to slow down the growth and spread of tumors. Although chemotherapy kills fast-dividing cancer cells as well as fast-dividing normal cells, it is able to inhibit cancer metastasis to some extent. However, the severe toxicity of chemotherapy is intolerable to most patients. It has been long

thought that the patient's own immune defense system is the best way to fight cancer metastasis. At the present, the most commonly used immunotherapies can be divided into three categories: (1) immunity manipulation through administration of cytokines such as interleukins, interferons, etc; (2) immunotherapy with monoclonal antibodies specifically against one or several cancer related antigens ("CRA's"); and (3) vaccination with CRA's (Ying et al. (2001) Innovative cancer vaccine strategies based on the identification of tumor-associated antigen. *BioDrugs* 15:819-31).

[0011] Immunity manipulation. Interferons belong to a group of proteins known as cytokines. They are produced naturally by white blood cells in the body (or in the laboratory) in response to infection, inflammation, or stimulation. Interferon-alpha was one of the first cytokines to show an anti-tumor effect, and it is able to slow tumor growth directly, as well as help to activate the immune system. Interferon-alpha has been approved by the FDA and is now commonly used for the treatment of a number of cancers, including multiple myeloma, chronic myelogenous leukemia, hairy cell leukemia, and malignant melanoma. Interferon-beta and interferon-gamma are other types of interferons that have been investigated. Other cytokines with anti-tumor activity include the interleukins (e.g., IL-2) and tumor necrosis factor. IL-2 is frequently used to treat kidney cancer and melanoma. Since cytokines regulate cascades of specific immune responses rather than directly manipulate the immune system to specifically fight cancer, undesirable side effects are commonly observed when cytokines are used to treat cancer. Some of the problems with these cytokines, including many of the interferons and interleukins, are their side effects, which include malaise and flu-like syndromes. When given at a high dose, the side effects can be greatly magnified.

[0012] Monoclonal antibodies. Another important biological therapy involves antibodies against cancer cells or cancer-associated targets. Monoclonal antibodies are artificial antibodies against a particular target (the "antigen") and are produced in the laboratory. The original method involved hybridoma cells (a fusion of two different types of cells) that acted as factories of antibody production. A major advance in this field was the ability to convert these antibodies, which originally were made from mouse hybridoma cells, to "humanized" antibodies that more closely resemble our natural antibodies. Even newer techniques can be used to generate human antibodies from genetically engineered mice or bacteria containing human antibody genes. Monoclonal antibodies have been widely used in

scientific studies of cancer, as well as in cancer diagnosis. As therapy for cancer, monoclonal antibodies can be injected into patients to seek out the cancer cells, potentially leading to disruption of cancer cell activities or to enhancement of the immune response against the cancer. This strategy has been of great interest since the original invention of monoclonal antibodies in the 1970's. After many years of clinical testing, researchers have shown that improved monoclonal antibodies can be used effectively to help treat certain cancers. An antibody called rituximab ("Rituxan") can be useful in the treatment of non-Hodgkin's lymphoma, while trastuzumab ("Herceptin") is useful against certain breast cancers. Other new monoclonal antibodies are undergoing active testing. However, one of the draw backs of using monoclonal antibodies for specific types of cancer related antigens ("CRA's") is that the types of CRA's and the amount of each type of CRA's can vary from one patient to another. Even for the same patient, the types of CRA's and the amount of each type of CRA's in the different developmental stages may be distinct. Accordingly, there are at least two drawbacks to treat cancer with monoclonal antibodies. Firstly, the efficacy is compromised if only a few of the CRA's are targeted with monoclonal antibodies. This is a particular drawback since most cancers are believed to be a multi-gene related. Secondly, different patients have different CRA's, and one or a group of specific monoclonal antibodies only will be effective for a limited number of cancer patients.

[0013] Cancer Vaccine. Although immunotherapies such as interferon and monoclonal antibodies have become part of standard cancer treatment, many other types of immunotherapy, such as cancer vaccines, remain experimental. In general, vaccines have revolutionized public health by preventing the development of many important infectious diseases, including polio, small pox, and diphtheria. However, it has been much more difficult to develop effective vaccines to prevent cancer, or to treat patients who have cancer. Despite many decades of experimental work, the attempts to develop cancer vaccines have not yielded successful results. In spite of this, a notable increase in interest has been generated by recent advances in the areas of immunology and cancer biology, which have led to more sophisticated and promising vaccine strategies than those previously available. At present, there are three basic strategies to make a cancer vaccine: (1) vaccination with one or a group of cancer related antigens ("CRA"); (2) to vaccinate a patient with dendritic cells ("DC's") pulsed with cancer tissue lysate of the same patient; (3) to vaccinate a patient with complexes of heat shock proteins ("HSP's") and CRA's isolated from the same patient.

[0014] (1) Vaccination with CRA. Cancer vaccines typically consist of a source of cancer related antigen ("CRA"), along with other components that further stimulate the immune response against the CRA. The challenge has been to find a better CRA, as well as to package the antigen in such a way as to enhance the patient's immune system to fight cancer cells that have the CRA. Increasingly, cancer vaccines have been shown to be capable of improving the immune response against particular antigens. The result of this immunologic effect is not always sufficient to reverse the progression of cancer. However, cancer vaccines have been generally well tolerated, and they may provide useful anticancer effects in some situations. For example, in malignant lymphoma, a number of laboratory studies have indicated that vaccination using lymphoma-associated proteins called "idiotype" can stimulate the immune systems of mice sufficiently to help them resist the development of lymphomas. In clinical trials, idiotype vaccines continue to be tested and have been associated with indications of clinical benefit in some lymphoma patients. In malignant melanoma, a wide variety of vaccine strategies have been introduced into clinical trials, and some have been found to stimulate the immune response against the cancer.

[0015] The disadvantages to vaccinate patients with one or a group of CRA's are the same as using monoclonal antibodies to treat cancer: (1) the efficacy is compromised if only a few of the CRA's are targeted; and (2) different patients have different CRA's, and (3) the resultant vaccines only will be effective for a limited number of cancer patients.

[0016] (2) Vaccination with dendritic cells ("DC's") pulsed with cancer tissue lysate. The many new strategies for vaccine construction and immune stimulation may lead to the emergence of clinically useful cancer vaccines. An example of one exciting new approach being tested in melanoma and other cancers is the use of dendritic cell vaccines. Dendritic cells ("DC") help to "turn on" the immune response. A dendritic cell is a type of antigen presenting cell ("APC") characterized by its potent capacity to activate naive T cells (Banchereau,J. et al. (2000) Immunobiology of dendritic cells. *Annu. Rev.Immunol.*18:767-81). By administration with DCs pulsed by CRA's in experimental animals, the cancers of these animals were diminished (Fong and Engleman (2000) Dendritic cells in cancer immunotherapy. *Annu.Rev.Immunol.*18:245-273). Similar results have been demonstrated for human patients (Nestle et al. (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat.Med.* 4:328-332). DC's also can be fused to cancer cells and the CRA's are pulsed into the DCs (Gong et al.(1997) Induction of anti-tumor activity by

immunization with fusion of dendritic and carcinoma cells. *Nat.Med.*3:558-561). It has been exhibited that DC's pulsed with CRA's have the ability to suppress metastatic cancers (Kugler (2000) et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell. *Nat.Med.* 6:332-336). This vaccination technology is a four-step process: (1) isolation of DC's from a patient and proliferation of the isolated DC's *ex vivo*; (2) *ex vivo* manipulation of DC's maturational state; (3) *ex vivo* incubation of DC's with CRA's from the same patient; (4) infusion of the DC's pulsed by CRA's back to the same patient. Thus, vaccination with DC's pulsed by cancer tissue lysate from the same patient has great potential to be extremely effective to treat local cancer as well as metastatic cancer with a low risk of detrimental toxicities. Because each individual patient's whole set of CRA's are presented to the same patient's immune system, such vaccines have been called "individualized vaccines". However, the disadvantages of individualized vaccines are (1) high-cost, (2) time-consuming, (3) sophisticated and tedious protocols of *ex vivo* preparation, that is often interrupted by contaminations, and (4) necessary to customize the vaccine for each individual patient, (*i.e.*, impossible to develop a drug based on the concept of these individualized vaccines.) (Srivastava and Jaikaria (2001) Methods of purification of heat shock protein-peptide complexes for use as vaccines against cancers and infectious diseases. *Methods Mol. Biol.* 156:175-186).

[0017] (3)Vaccination with CRA complexed with Heat Shock Proteins ("HSP's"). The elevated expression of a group of heat shock proteins ("HSP's"), or stress proteins by any environmental stimulus including physical, chemical and biological stimuli is defined as a heat shock response or stress response. Srivastava *et al.* found that (1) heat shock proteins, HSP70 in particular, can bind episode peptides of cancer specific proteins to form complexes, and these complexes can be purified *ex vivo*; (2) infusion of these purified complexes results in that the episode peptides as CRA's complexed with HSP's migrate to DC's *in vivo*; (3) DC's present these CRA's to the immune system and induce immunity against cancer (Basu and Srivastava (2000) Heat shock proteins: the fountainhead of innate and adaptive immune responses. *Cell Stress & Chaperones* 5:443-451).

[0018] Haviv *et.al.* reported that HSP70 is capable to enhance the ability of oncolytic viruses to kill cultured cancer cells (Haviv et al.(2001) Heat shock and Heat shock protein 70i enhance the oncolytic effect of replicative Adenovirus. *Cancer Research* 61:8361-8365). However, their *in vitro* tests can not determine whether HSP70 may enhance the

efficacy of oncolytic viruses to treat cancer without damaging the normal biological functions of animal or human. Furthermore, due to that they only did their experiments on cultured cancer cells (lung cancer lines A549, H460, and H157), they were not able to demonstrate that viral oncolysis of the cancer cells that contain high level of HSP70 would induce a systemic immune response against cancer, and consequently to treat local and metastatic cancers.

[0019] It is tempting to develop a single agent that may lead to the presentation of every cancer patient's complete set of CRA's to his/her own immune system, and induce immunity against cancer accordingly. Recently, Chen and Hu developed a viral agent that can present the whole set of almost every cancer patient's CRA's to his/her own immune system, and induce immune response against his/her own cancer (China Patent Application 01141696.3 & Pct/cn01/01616). Animal tests have demonstrated that this viral agent is effective to inhibit the growth of the treated tumor. This viral agent is an oncolytic adenovirus carrying an exogenous HSP70 gene. Although not wanting to be bound by theory, the oncolytic viruses can lyse cancer cells, and the HSP70 expressed by the viruses can capture CRA's. Following the lysis of the cancer cells that have been infected by oncolytic viruses, the CRA's complexed with HSP70 are presented to DCs, and subsequently elicit immune response against cancer cells. Although not wanting to be bound by theory, the heat shock response is a complex multi-step process, wherein HSP70 may only be one critical protein in the pathway responsible for proper presentation of the complexed CRA-HSP. Consequently, it may be necessary induce the entire set of heat shock proteins such as HSP60, HSP70, HSP90, HSP110 and so on in a treated tissue to get an adequate immune response to successfully treat metastatic cancers.

[0020] **II. Currently available techniques to elevate the expression of endogenous HSP's:** Although not wanting to be bound by theory, there are several known environmental stimuli that can induce a heat shock cascade in order to increase the endogenous expression of HSP. These stimuli include hyperthermia, alcohol, inhibitors of energy metabolism, heavy metals, oxidative stress, inflammation, etc (Zylicz et al.(2001) HSP70 interactions with the p53 tumor suppressor protein. *The EMBO Journal* 20:4634-4638). A correlation between a feverish infection and a concurrent remission from cancer has been observed, and recent publications attribute this correlation to the expression of HSP (Hobohm (2001) Fever and cancer in perspective. *Cancer Immunol Immunother.* 50: 391-396). Other non-toxic chemicals such as glutamine and amino acid analogs can also elevate

the expression level of HSPs (Wischmeyer (2002) Glutamine and Heat Shock Protein expression. *Nutrition* 18:225-228; van Rijn et al.(2000) Heat shock responses by cells treated with azetidine-2-carboxylic acid. *Int J Hyperthermia* 16:305-318). In addition, mitochondrion uncoupling agents such as albendazole raise body temperature, and hence increase the expression of HSP's (Wallen et al.(1997) Oxidants differentially regulate the heat shock response. *Int J Hyperthermia* 13:517-24).

[0021] In summary, prior art has shown that it is possible to treat cancer conditions in a limited capacity utilizing various technologies and treatments, however, many of these treatments have some significant drawbacks. One aspect of the invention described herein relates to a well-timed hyperthermia applied to a malignant tumor wherein a genetically engineered oncolytic virus has also been administrated, the combination of heat and virus elicits an immune response directed against the cancer. Consequently, the combination of hyperthermia and viral oncolysis is effective to suppress the locally treated tumor as well as the distal not-treated metastasis. Another aspect of the current invention is related to other stimuli (e.g. physical, chemical or biological) that elevate the endogenous expression of HSP's in combination with viral oncolysis, wherein local treatment decrease local and distal tumors.

SUMMARY

[0022] Broadly, the present invention relates to compositions and methods for ablating tumor cells in a subject having at least one tumor site. More specifically, the method comprises contacting the tumor cells in at least one tumor with a lytic agent in vivo, under lytic conditions, forming a treated tumor; and applying a sufficient in vivo stimulus to the treated tumor forming a stimulated tumor.

[0023] One aspect of the current invention is a method for shrinking a tumor in a subject comprising the steps of: introducing a lytic agent into the tumor; once a maximum process of lysis has occurred, a stimulus is then applied to the tumor for a first period of time. The stimulus that is applied to the tumor can normally elevate the level of heat shock proteins ("HSP's") in the tumor. The first period of time is generally about 15 minutes to 90 minutes. In a preferred embodiment, a method for shrinking a tumor includes the following method steps: (1) introducing a lytic agent into a tumor for a first number of rounds (e.g. about 1-10 rounds); (2) applying a stimulus to the tumor for a first period of time (e.g. about 15-90 minutes) starting from the second day after the first introduction of lytic agent, that can be repeated every day for a second number of rounds (e.g. about 1-20 rounds).

[0024] The method described herein can be applied to specific types of tumors. Although not wanting to be bound by theory, tumors that consist of a defective tumor-suppressor gene (e.g. defective p53), an activated oncogene (e.g. ras, or myc) are good candidates for this method of therapy. Exemplified by, but not limited to, the invention described herein is useful for a nasopharyngeal carcinoma, a breast cancer, a prostate cancer, an ovarian cancer, a malignant hepatoma, a carcinoma of esophagus, a lung cancer, a cancer of rectum, a carcinoma of stomach, a carcinoma of ovarium, a ascites, or a melanoma. In specific embodiments, the lytic agent comprises either an oncolytic virus (e.g. an adenovirus, a herpes simplex virus, a reovirus, a Newcastle disease virus, a poliovirus, a measles virus, or a vesicular stomatis virus), or an oncolytic bacterium (e.g. *Salmonella, Bifidobacterium, Shigella, Listeria, Yersinia,* or *Clostridium*), or an any type of oncolytic agent. The oncolytic virus/oncolytic bactierium can be either wild-type or genetically engineered form. Additionally, the lytic agent may comprises a therapeutic gene (e.g. an apoptotic gene, a gene for tumor necrosis, a gene for starving tumor cells to death, cytolytic gene, negative I-κ-β, caspase, γ globulin, hα-1 antitrypsin, or E1a of adenovirus).

[0025] The method step of stimulating the tumor includes: local hyperthermia; systemic hyperthermia; a high-frequency electromagnetic pulses; radiofrequency diathermy; ultrasound diathermy; an anoxia, a radiation, an alcohol, a glutamine, an infection, or an any kind of physical, chemical or biological stimulus. In a specific embodiment, local hyperthermia, is in the range of about 1 to about 7 degrees Celsius above a normal body temperature of the subject. Generally, the stimulus elevates heat shock proteins (e.g. Hsp30, Hsp60, Hsp70, Hsp90, Hsp94, Hsp96, or Hsp110) in the stimulated tumor. By following the method of this invention, the shrinking of a tumor in a subject can be accomplished.

[0026] Another aspect of the current invention is a method for shrinking a "not-treated tumor" (or a metastasis) in a subject comprising the steps of: introducing a lytic agent into a tumor (a "treated tumor"). Once a process of lysis has occurred, a stimulus is then applied to the treated tumor. The stimulus that is applied to the treated tumor is capable of elevating the level of heat shock proteins ("HSP's") in the treated tumor. In a preferred embodiment, a method for shrinking a not-treated tumor includes the following method steps: (1) introducing a lytic agent into a tumor (the treated tumor) for a first number of rounds (e.g. about 1-10 rounds); (2) applying a stimulus to the treated tumor for a first period of time (e.g. about 15-90 minutes) starting from the second day after the first introduction of lytic agent, that can be repeated every day for a second number of rounds (e.g. about 1-20 rounds). Not wanting to be bound by theory, the specific immunity elicited by the synchronization of introducing a lytic agent and applying a stimulus shrinks the not-treated tumors. The method described herein has been contemplated by the inventors to be applied to specific types of distal-tumors. Not wanting to be bound by theory, the treated or not-treated tumors that consist of a defective p53 tumor-suppressor gene (e.g. a defective p53), an activated oncogene (e.g. ras, or myc) are good candidates for this method of therapy. Exemplified by, but not limited to, the invention described herein is useful for a nasopharyngeal carcinoma, a breast cancer, a prostate cancer, an ovarian cancer, a malignant hepatoma, a carcinoma of esophagus, a lung cancer, a cancer of rectum, a carcinoma of stomach, a carcinoma of ovarium, a ascites, or a melanoma. In specific embodiments, the lytic agent comprises either an oncolytic virus (e.g. an adenovirus, a herpes simplex virus, a reovirus, a Newcastle disease virus, a poliovirus, a measles virus, or a vesicular stomatis virus), or an oncolytic bacterium (e.g. *Salmonella, Bifidobacterium, Shigella, Listeria, Yersinia,* or *Clostridium*), or an any type of oncolytic agent. The oncolytic virus/oncolytic bactierium can be either wild-type or genetically

engineered form. Additionally, the lytic agent may comprises a therapeutic gene (e.g. an apoptotic gene, a gene for tumor necrosis, a gene for starving tumor cells to death, cytolytic gene, negative I-κ-β, caspase, γ globulin, hα-1 antitrypsin, or E1a of adenovirus).

[0027] The method step of stimulating the first-tumor was contemplated by the inventors to include: local hyperthermia; systemic hyperthermia; a high-frequency electromagnetic pulses; radiofrequency diathermy; ultrasound diathermy; an anoxia, a radiation, an alcohol, a glutamine, an infection, or an any type of stimulus. In a specific embodiment, local hyperthermia, is in the range of about 1 to about 7 degrees Celsius above a normal body temperature of the subject. Generally, the stimulus elevates heat shock proteins (e.g. Hsp30, Hsp60, Hsp70, Hsp90, Hsp94, Hsp96, or Hsp110) in the stimulated tumor. By following the method of this invention, the shrinking of a not-treated tumor in a subject can be accomplished.

BRIEF DESCRIPTION OF FIGURES

[0028] Figure 1 shows the illustration of the genetically modified S98 adenoviruses;

[0029] Figure 2 shows the replication of the genetically modified S98 adenoviruses in normal cells, wherein MOI abbreviates multiplicity of infection;

[0030] Figure 3 shows an intratumoral injection dosage escalation curve for the 5 dose levels utilized for H101 (SEQID#1); and

[0031] Figure 4 shows the number and types of tumor patients enrolled in study to determine a dosage escalation curve.

DETAILED DESCRIPTION:

[0033] All of the terms used herein refer to the definitions commonly agreed by the scientific community. To insure that the terms used herein are not misinterpreted, the definitions of these terms are given as following:

[0034] The term "adjuvant" as used herein refers to a substance that can be used together with antigens, or itself can be used as antigen to elicit immunity.

[0035] The term "antigen" as used herein refers to a kind of substances that elicit immune responses, including antibody generation, activation of specific immunological cells, or the combination of the two. Antigens could be a biological macro-molecule, part of a biological macro-molecule, debris of organism, etc. .

[0036] The term "antigen presentation cell" as used herein refers to a kind of cell whose function is to process and present antigens to T cell and B cell. This type of cells includes dendritic cell, macrophage cell and B cell.

[0037] The term "cancer" as used herein refers to malignant tumor that metastasize and proliferate immortally. Cancer is a group of diseases classified by the tissues affected, and include, but are not limited to breast cancer, prostate cancer, ovarian cancer, malignant hepatoma, carcinoma of esophagus, lung cancer, cancer of rectum, nasopharyngeal carcinoma, carcinoma of stomach, pleural effusion, carcinoma of ovarium, ascites, and melanoma.

[0038] The term "cancer gene therapy" as used herein refers to that vectors carrying therapeutic gene(s) infect cancer cells, so as to destroy cancer cells. The therapeutic genes include genes related to cell apoptosis, cell lysis, cell suicide, etc. These therapeutic genes also include negative i-κ-βgene, caspase gene, γ-globulin gene, α-1 anti-trypsin gene, E1a gene for oncolytic adenovirus, etc..

[0039] The term "cancer related antigen" as used herein refers to antigen that represents the unique characteristics of cancer cells. Cancer related antigen is abbreviated as CRA.

[0040] The term "cancer vaccine" as used herein refers to a CRA or immunological cells that have encountered with CRA's. A CRA could be a molecule representing the unique characteristics of cancer cells or an episode of this type of molecule. In well-manipulated compositions, cancer vaccine may elicit patient's immunity against cancer.

[0041] The term "chaperones" as used herein refer to a group of unrelated proteins that mediate the correct folding, assembly, reparation, translocation across membranes and degradation of other proteins and simultaneously are not their functional components. One embodiment describes the "Hsp70" multi-gene family as one type of chaperones. The advantages to certain types of chaperones are characterized in specific embodiments of the invention, but they are not intended to be limiting.

[0042] The term "exogenous gene" or "trans-gene" as used herein refers to DNA sequences encoding a protein of interest inserted into a vector of gene therapy at a specific location. Exogenous gene could be from the vector itself, but had been rearranged on the genome of the vector. However, exogenous gene more often is a DNA fragment from the genome of another organism. The sequence of exogenous gene may be prepared by chemical/biochemical synthesis, by purification from a natural source, by cloning, or by any other methods.

[0043] The term "heat shock protein" as used herein refers to a family of proteins expressed universally in almost all kinds of organisms from bacteria to human. They are also named as "stress proteins" and abbreviated as HSP's. The expression of HSP's are regulated by environmental stimuli and developmental influences, e.g., hyperthermia, anoxia, alcohol, glucose starvation (for glucose regulated proteins, or GRP's, that are also a sub-group of HSP's), tissue injury, infection, etc. HSP's play crucial roles in protein folding and protein metabolism. They may transport immunogens to DC cells that have receptors on cell membrane for HSP's. The heat shock proteins with an elevated expression level, either individually or in combination, after hyperthermic treatment include but are not limited to Hsp30, Hsp60, Hsp70, Hsp90, Hsp94, Hsp96, and Hsp110.

[0044] The term "Hsp70" as used herein refers to a multi-gene family of chaperones, but all members have a four common features: highly conserved sequence,

molecular mass about 70 kDa, ATPase activity and an ability to bind and release of hydrophobic segments of unfolded polypeptide chains.

[0045] The term "lytic agent" as used herein refers a composition capable of rupturing a tumor cell.

[0046] The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. As used herein, the term "recombinant" indicates that a polynucleotide construct (e.g., and adenovirus genome) has been generated, in part, by intentional modification by man.

[0047] The term "not-treated tumor" as used herein refers to a tumor where oncolytic agents and environmental stimuli elevating the expression of HSP's are NOT applied directly, regardless if it is a primary tumor or a metastatic tumor. The not-treated tumor may be remote from the site of the application of oncolytic agent and the environmental stimuli elevating the expression of HSP's. The term "distal-tumor" can also be utilized interchangeably.

[0048] The term "oncolytic bacterium" as used herein refers to a genetically engineered bacterium that may replicate immortally in cancer cells, so as to kill these cancer cells. *Salmonella typhimurium* YS72, *Bifidobacterium, Shigella, Listeria, Yersinia, Clostridium* are examples, other examples are described in the article by Bermudes et al. (Bermudes et al. (2002) Live bacteria as anticancer agents and tumor-selective protein delivery vectors. *Curr Opin Drug Discov Devel.* 5(2):194-9.), the entire content is herein incorporated by reference.

[0049] The term "oncolytic techniques" as used herein refers to all kinds of effective protocols that can induce the lysis or death of tumor cells including apoptosis and necrosis. These protocols include application of oncolytic virus, oncolytic bacteria and any other agents that lead to the lysis or death of cancer cells.

[0050] The term "oncolytic virus" as used herein refers to a genetically engineered virus that may replicate immortally in cancer cells, so as to kill these cancer cells. Adenovirus *dl*1520 is an example of oncolytic viruses.

[0051] The term "p53 function" As used herein refers to the property of having an essentially normal level of a polypeptide encoded by the p53 gene (i.e., relative to non-neoplastic cells of the same histological type), wherein the p53 polypeptide is capable of binding an E1b p55 protein of wild-type adenovirus. For example, p53 function may be lost by production of an inactive (i.e., mutant) form of p53 or by a substantial decrease or total loss of expression of p53 polypeptide(s). Also, p53 function may be substantially absent in neoplastic cells, which comprise p53 alleles encoding wild-type p53 protein. For example, a genetic alteration outside of the p53 locus, such as a mutation that results in aberrant subcellular processing or localization of p53 (e.g., a mutation resulting in localization of p53 predominantly in the cytoplasm rather than the nucleus) can result in a loss of p53 function.

[0052] The terms "percentage of sequence identity" as used herein compares two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e. "gaps") as compared to a reference sequence for optimal alignment of the two sequences being compared. The percentage identity is calculated by determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window and multiplying the result by 100 to yield the percentage of sequence identity. Total identity is then determined as the average identity over all of the windows that cover the complete query sequence. Although not wanting to be bound by theory, computer software packages such as GAP, BESTFIT, BLASTA, FASTA and TFASTA can also be utilized to determine sequence identity.

[0053] The term "RB function" as used herein refers to the property of having an essentially normal level of a polypeptide encoded by the RB gene (i.e., relative to non-neoplastic cells of the same histological type), wherein the RB polypeptide is capable of binding an E1a protein of wild-type adenovirus. For example, RB function may be lost by production of an inactive (i.e., mutant) form of RB or by a substantial decrease or total loss of expression of RB polypeptide(s). Also, RB function may be substantially absent in neoplastic

cells that comprise RB alleles encoding a wild-type RB protein. For example, a genetic alteration outside of the RB locus, such as a mutation that results in aberrant subcellular processing or localization of RB, may result in a loss of RB function.

[0054] The term "replication deficient virus" as used herein refers to a virus that preferentially inhibits cell proliferation or induces apoptosis in a predetermined cell population (e.g., cells substantially lacking p53 and/or RB function) which supports expression of a virus replication phenotype, and which is substantially unable to inhibit cell proliferation, induce apoptosis, or express a replication phenotype in cells comprising normal p53 and RB function levels characteristic of non-replicating, non-transformed cells. Typically, a replication deficient virus exhibits a substantial decrease in plaquing efficiency on cells comprising normal RB and/or p53 function.

[0055] The term "replication phenotype" as used herein refers to one or more of the following phenotypic characteristics of cells infected with a virus such as a replication deficient adenovirus: (1) substantial expression of late gene products, such as capsid proteins (e.g., adenoviral penton base polypeptide) or RNA transcripts initiated from viral late gene promoter(s), (2) replication of viral genomes or formation of replicative intermediates, (3) assembly of viral capsids or packaged virion particles, (4) appearance of cytopathic effect (CPE) in the infected cell, (5) completion of a viral lytic cycle, and (6) other phenotypic alterations which are typically contingent upon abrogation of p53 or RB function in non-neoplastic cells infected with a wild-type replication competent DNA virus encoding functional oncoprotein(s). A replication phenotype comprises at least one of the listed phenotypic characteristics, preferably more than one of the phenotypic characteristics.

[0056] The terms "S-98" and "H101" can be used interchangeably.

[0057] The term "stimulus" as used herein refers any action or agent that causes or changes an activity in an organism, organ, cell, or part thereof. In general, the stimulus described in specific embodiments are "in addition" to any change or impulse resulting from the introduction of the lytic agent to the tumor cells. One embodiment described herein utilizes an external hyperthermia as the stimulus. Another embodiment described herein utilizes systemic hyperthermia as the stimulus. In yet another embodiment, the stimulus utilized increases the level of chaperone proteins in the tumor cells. The advantages to

certain types of stimulus are characterized in specific embodiments of the invention, but they are not intended to be limiting.

[0058] The term "treated tumor" as used herein refers to a designated tumor where oncolytic agents and environmental stimuli elevating the expression of HSP's are directly applied, no matter it is a primary tumor or a metastatic tumor. In some embodiments, the "first-tumor" is synonymous with the treated-tumor.

[0059] The term "T-lymphocyte" as used herein refers to a kind of cell that derived from thymus and can participate in a series of immune response.

[0060] The present invention relates generally to compositions and methods for ablating tumor cells in a subject having at least one tumor site. More specifically, the method comprises contacting the tumor cells in at least one tumor with a lytic agent in vivo, under lytic conditions, forming a treated tumor; and applying a sufficient *in vivo* stimulus to the treated tumor forming a stimulated tumor. Although not wanting to be bound by theory, the stimulated tumor expresses at least one chaperone protein at an elevated level compared to that of the tumor prior to applying the stimulus. The chaperone protein may comprises a heat shock protein ("HSP") that binds a CRA from a lysed tumor cell and presents the CRA to the subject's immune system, whereby alerting the subject's immune system to the presence of a growing tumor.

[0061] The present invention relates to the synchronization between different kinds of oncolysis and different techniques to elevate expression of HSPs. To be more specific, this invention relates to: (1) oncolysis by a virus, a bacterium, or an any kind of agent at a designated cancer; (2) timely application of any kind of physical, chemical, or biological stimulus, *e.g.*, hyperthermia, glutamine that elevates the expression of HSP's to the tumor where oncolytic agent was administrated so that enough HSP's capture enough CRA's to form HSP-CRA complexes; (3) the synchronization of elevated expression of HSP's and oncolysis results in sufficient release of HSP-CRA where released CRA's accurately represent the complete set of a patient's CRA's; (4) the sufficient amount of HSP-CRA is then autogenously exhibited to DC cells, and is eventually presented to the immune system; (5) the signal of HSP-CRA presented to the immune system is immunogenic enough to elicit immune response against cancer; (6) this immunological treatment for cancer can be used for both the

treated tumor and the not-treated tumor, no matter whether it is a primary or metastatic cancer.

[0062] **The oncolytic techniques.** The development of novel cancer therapies that are selective for cancer cells with limited toxicity to normal tissues is a challenge for oncology researchers. Microorganisms, such as viruses with selectivity for tumor cells or tumor micro-environments, have been investigated as potential arsenals for decades. Genetically-modified, non-pathogenic bacteria have begun to emerge as potential antitumor agents, either to provide direct tumoricidal effects or to deliver tumoricidal molecules. Attenuated *Salmonella, Clostridium and Bifidobacterium* are capable of multiplying selectively in tumors and inhibiting their growth, representing a new approach for cancer treatment. Because of their selectivity for tumor tissues, these bacteria would also be ideal vectors for delivering therapeutic proteins to tumors. VNP20009, an attenuated strain of *Salmonella typhimurium*, and its derivative, TAPET-CD, which expresses an Escherichia coli cytosine deaminase (CD), are particularly promising, and are currently undergoing phase I clinical trials in cancer patients. (Bermudes et al. (2002) Live bacteria as anticancer agents and tumor-selective protein delivery vectors. *Curr Opin Drug Discov Devel.* 5(2):194-9.). Other examples of oncolytic bacteria can be exemplified by, but not limited to *Salmonella, Bifidobacterium, Shigella, Listeria, Yersinia, and Clostridium.*

[0063] Any viruses, bacteria, or other agents that may selectively replicate in cancer cells can be used for the purpose of oncolysis. The oncolytic viruses referred to in this invention could be herpes simplex virus (HSV-1), adenovirus, newcastle disease virus ("NDV"), poliovirus, measles virus, vesicular stomatitis virus ("VSV"), etc.

[0064] Although not wanting to be bound by theory, prior reports have demonstrated that mutation of *p*53 gene is one of the most common gene mutations for cancer patients. Mutations of *p*53 gene exist in more than half of cancer cases. One of the oncolytic techniques targeting cancers with mutation on this gene is the oncolytic virus modified from human Ad5 adenovirus with alteration in E1b region that encodes the protein E1b-55KD. This oncolytic adenovirus selectively replicates in cancer cells with *p*53 gene mutation, thus lyse cancer cells with high specificity. Two variant Ad5 viruses S98-001 (SEQID#1) and S98-002 (SEQID#2) with alteration in E1b region encoding for protein E1b-55kd are used as examples in this invention.

[0065] In addition, when selectively delivered to cancer cells by proper vectors, many therapeutic genes exemplified by, but not limited to genes for apoptosis, genes for cytolysis, genes for tumor necrosis, genes for starving tumor cells to death, negative I-κ-βgene, caspase gene, γ globulin gene , hα-1 anti-trypsin gene, E1a gene of adenovirus, etc may be used for the purpose of oncolysis.

[0066] **The techniques to elevate expression of HSP's.** Although not wanting to be bound by theory, it has been demonstrated that local hyperthermia and whole-body hyperthermia may elevate the expression of HSP's in human and animals (Li et al. (1995) Heat shock proteins, thermotolerance, and their relevance to clinical hyperthermia. *Int. J. Hyperthermia* 11(4): 459-488). Accordingly, local hyperthermia (temperature range: 38°C to 45°C) and whole-body hyperthermia (body temperature below 42°C) for 5 to 90 minutes were used in synchronization with oncolysis to treat local and metastatic cancers.

[0067] High-frequency electromagnetic radiation such as radiofrequency (0.1-100MHz) diathermy and microwave (100-2,450MHz) diathermy is most frequently used for local hyperthermia, due to its high efficiency, deep penetration, easily controlled dosage and simplicity to operate. Radiofrequency diathermy is suitable for deep-seated tumors, and microwave diathermy suits for superficial tumors. In addition, ultrasound diathermy can be used for both superficial and deep-seated tumors, though it is not appropriate for most tumors involving bone or behind gas-filled cavities, such as bowel or lung. It is noteworthy that, for this invention, hyperthermia is not used to kill local and distal cancer cells directly, but to induce the higher expression of HSP's. Additionally, the hyperthermic techniques chosen in this invention should have no impediments for the oncolytic efficiencies of oncolytic microorganisms such as oncolytic viruses, oncolytic bacteria and other vectors for gene therapy.

[0068] Although not wanting to be bound by theory, other alternatives to increase the expression of HSP's are exemplified by, but not limited to anoxia, radiation, alcohol, certain inhibitors of energy metabolism, glutamine, and any other agents that is able to elevate local or whole-body temperature and is safe to human. Any biological means that may up-regulate the expression of HSP's, *e.g.*, heat shock transcriptional factors, infections, etc, also potentially can be used in synchronization with oncolysis to elicit immunity against cancer.

[0069] **Synchronization of oncolysis and elevated expression of HSP's.** The implementation protocol of this invention can be any synchronization of the above two techniques. One of the techniques to elevate the expression of HSP's synchronized with one or multiple oncolytic techniques will elicit immune response against cancer cells in order to treat primary and metastatic cancers.

[0070] One aspect of an optimized treatment for primary and metastatic cancers comprises the synchronization of hyperthermia and oncolysis by a variant adenovirus with E1b-55 KD alterations. Hyperthermia increases the expression of HSP's, and the variant adenovirus with E1b-55KD alterations lyses cancer cells selectively. When an oncolytic adenovirus lyses cancer cells at a high level, the amount of functional HSP's should also be at a high level. Only if these two "high levels" are synchronized, enough HSP-CRA's will exhibit a signal immunogenic enough to the immune system in order to elicit the immune response against cancer.

[0071] Although not wanting to be bound by theory, it has been demonstrated that (1) the optimum conditions to increase the expression of HSP's is in the temperature range of 38℃ to 45℃ and the time range of 15 to 90 minutes (Li and Mak (1985) Induction of heat shock protein synthesis in murine tumors during the development of thermotolerance. *Cancer Res.* 45(8):3816-3824); (2) the elevated expression of HSP's starts minutes after hyperthermic treatment ends, and the elevated level of HSP's can maintain for 24 to 48 hours (Li (1984) Thermal biology and physiology in clinical hyperthermia: current status and further needs. *Cancer Res. (Suppl.)* 44(8):48865-48935); (3) the inventors of this invention have determined that the maximum oncolytic effect of an oncolytic adenovirus occurs in 4 to 10 days after viral injection. Accordingly, the inventors have contemplated a protocol to maximally synchronize viral oncolysis and elevated expression of HSP's. The brief protocol comprises an outlined protocol: to inject an oncolytic adenovirus into a tumor, once a day for 5 days; and then to apply hyperthermia to the tumor of viral injection in the temperature range of 38℃ to 45℃ for 15 to 90 minutes. The hyperthermic treatment starts from the second day after the first viral injection and lasts for 8 to 16 days.

[0072] One aspect in this invention comprises an oncolytic adenovirus S98-001 (SEQID#1) with E1b-55 KD alterations that is injected into a tumor of a cancer patient, and radiofrequency diathermy (wave range at 4-24 μm, penetration range at 4-5 mm) was also

subjected to the same tumor in the temperature range of 38° to 45° for 15 to 90 minutes to control the growth of the treated tumor and the growth of the not-treated tumors.

[0073] To deliver a oncolytic agent or an agent elevating the expression of HSP's, the various routes, e.g., intratumoral injection, parenteral administrations including intramuscular, intravenous and subcutaneous injections, oral administration and other systematic administrations including transdermal administrations, intranasal administrations and through suppositories can be used. The compositions can be tablet, pill, capsule, semisolid, powder, sustained release preparation, solution, suspension, aerosol or any other suitable forms. Immunity against cancer can be elicited by a composition or a pharmaceutical formula that includes both an agent for oncolysis and an agent increasing the expression of HSP's, *e.g.*, a dosage form of an oncolytic virus and glutamine. A excipient used in the compositions can be any solid, liquid, semisolid, or gas in the presence of aerosol.

EXAMPLES

[0074] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow are presented by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

[0075] Generally, an adenovirus is in a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans or animals. The virus that causes the common cold is an adenovirus. The oncolytic viruses of this invention comprises genetically engineered adenovirus Ad5 variants. Specific engineered variants of Ad5 viruses are used for this invention and comprise S98-001 (SeqID#1) or S98-002 (SeqID#2). Although not wanting to be bound by theory, it is known that an infection of the human body with a wild-type Ad5 is autogenously curable. Additionally, the Ad5 adenovirus has been used routinely as a vector for gene therapy because there are no reports that the DNA

fragments of Ad5 genome can integrate into the genome of human cells. Thus, the synchronization of injecting a specific oncolytic virus and hyperthermia to inhibit cancer at the injection site and cancers distant from the viral injection site of are utilized in this invention. Although oncolytic Ad5 variants are used as specific examples, other lytic agent comprises either an oncolytic virus (e.g. an adenovirus, a herpes simplex virus, a reovirus, a Newcastle disease virus, a poliovirus, a measles virus, or a vesicular stomatis virus), or an oncolytic bacterium (e.g. *Salmonella, Bifidobacterium, Shigella, Listeria, Yersinia,* or *Clostridium*), or an any type of oncolytic agent. The oncolytic virus/oncolytic bactierium can be either wild-type or genetically engineered form. Additionally, the lytic agent may comprises a therapeutic gene (e.g. an apoptotic gene, a gene for tumor necrosis, a gene for starving tumor cells to death, cytolytic gene, negative I-κ-β, caspase, γ globulin, hα-1 antitrypsin, or E1a of adenovirus).

[0076] Genetically engineered adenovirus variants S98-001 (SEQID#1) and S98-002 (SEQID#2). The genome of a wild-type Ad5 (SEQID#3) is composed of about 35,935 bps. Genetically engineered mutants of Ad5 and variants having at least 95% homology with S98-001 (SEQID#1) and S98-002 (SEQID#2), which can replicate selectively in cancer cells are considered as examples for this invention (Figure 1). One distinction when comparing a wild-type Ad5 (SEQID#3) with the variant S98-001 (SEQID#1) is an extra TGA stop codon at position 2025 that is in E1b region of the variant. S98-001 (SEQID#1) also possesses two deletions: one is in E1b region between position 2,501 and position 3,328; the other is between position 27,865 and position 30,995 including the entire E3 region. A protein of 55 KD is encoded by the DNA sequence in E1b region. This protein is named as E1b-55 KD. In normal cells, E1b-55 KD binds and inactivates the protein encoded by the tumor-suppressor gene *p*53 so as to initiate virus replication. In S98-001 (SEQID#1), the two alterations in E1b region lead to the expression of a variant E1b-55 KD protein. This variant E1b-55 KD protein has very low binding affinity with *P*53 protein. Therefore, S98-001 (SEQID#1) is not able to replicate in normal cells. However, S98-001 (SEQID#1) replicates rapidly in cancer cells where *P*53 protein is dysfunctional. The function of E3 region is related to adenovirus' ability to escape from the surveillance of immune system. The complete deletion of E3 region in S98-001 (SEQID#1) enables the immune system easier to distinguish and eliminate this virus. Hence, S98-001 (SEQID#1) is less likely to infect and to lyse normal cells comparing to the variant Ad5 viruses that only have alteration in the E1b-55

KD region. It has been demonstrated that the ratio of cytolysis between cancer cells and normal cells is in the range of about 100:1 to about 1,000:1 for S98-001 (SEQID#1). Since S98-001 (SEQID#1) only lyses cancer cells, it is an oncolytic virus.

[0077] S98-002 (SEQID#2) is another genetically modified variant Ad5. S98-002 (SEQID#2) has two deletions: one in the region encoding E1b-55 KD, between position 2501 and position 3328; and the other between position 27,865 and position 30,995 including the entire E3 region. The purpose to prepare a Ad5 variant S98-002 (SEQID#2) demonstrates another embodiment of an oncolytic adenovirus can be generated. The variant DNA sequences of Ad5 are unable to integrate into human genome, but the Ad5 variants S98-001 (SEQID#1) and S98-002 (SEQID#2) selectively replicate in cancer cells. Therefore, S98-001 (SEQID#1) and S98-002 (SEQID#2) are safe for use in humans and animals.

[0078] **Preparation of oncolytic Ad5 variants. (1) Construction of S98 viruses.** pXC-1 and pBHG11 were purchased from Microbix Biosystem. pXC-1 contains the adenovirus type 5 (Ad5) sequence (bp22~5,790). PBHG11 contains the Ad5 sequence that has two deletions: bp188~1339 in E1 region which encodes the packaging signal of the viral capsid protein; and the deletion of E3 region (bp27,865~30,995). PBHG11 is not infective. However, co-transfection with pXC-1 and pBHG11 generates an infective virus based upon homologous recombination.

[0079] To amplify the Ad5 DNA fragment of bp1,338~2,501 on pXC-1 by a method of PCR, the following two oligonucleotide primers were used:

HZ1 (SeqID#4) (5'-CTATCCTGAGACGCCCGAC-3') and,

HZ2 (SeqID#5) (5'-GATCGGATCCAGGTCTCCAGTAAGTGGTAGCTGC-3'; with the BglII site underlined).

[0080] The synthesized DNA sequence was then cloned into vector pGEM-T (Promega) to obtain the plasmid HZ102. HZ103 was constructed by ligating HZ102 Xbal/Bgl II digested fragment to pXC-1 Xbal/Bgl II digested fragment. In order to create a stop codon on pXC-1 at bp2025, plasmid HZ104 was generated with Quick Change Site Directed Mutagenesis (Strategene). The two primers used in this step were:

HZ3 (SeqID#6) (5'-AAAGGATAAATGGAGTAAAGAAACC-3') and,

HZ4 (SeqID#7) (5'-CAGATGGGTTTGTTCATTTATCC-3').

[0081] The changed sequence of HZ104 had been confirmed by DNA sequencing. The HZ104 Xbal/Bgl II digested fragment was ligated to pXC-1 Xbal / Bgl II digested fragment to generate HZ105.

[0082] S98 viruses were generated using two overlapping plasmids by homologous recombination, then plaques were picked out and amplified in HYH cells. Since HYH expresses both E1A and E1B proteins normally, all of the S98 viruses can form plaques in HYH cells efficiently. Virus DNA was purified using QIAamp DNA Blood kit (Qiagen) and was analyzed by PCR and Southern blot.

[0083] Co-transfection of cell line 293 with pBHG11 and HZ105 generated S98-001 (SEQID#1). Co-transfection with pBHG11 and HZ103 generated S98-002 (SEQID#2), and co-transfection with pBHG11 and pXC-1 generated S98-100.

[0084] S98-100 has no alterations in E1b region so that it expresses E1b-55KD normally though its E3 region has been deleted. Consequently, S98-100 replicate as same as a wild-type adenovirus, *i.e.*, S98-100 not only replicate in cancer cells, but also in normal cells. Thus, S98-100 should be considered as a wild-type S98. S98-100 was used as the positive control to determine the oncolytic specificity for S98 viruses

[0085] Two alterations in E1b region, an extra TGA stop codon at position 2025 and a deletion between position 2501 and position 3328, makes S98-001 (SEQID#1) missing part of the DNA sequence coding for protein 495R (protein E1b-55 KD) and for protein 495R synthesis related mRNA-13S, 14S and 14.5S. In the other hand, the deletion in E1b region between position 2501 and position 3328 makes S98-002 (SEQID#2) missing part of the DNA sequence coding for protein 495R (protein E1b-55 KD). Thus, S98-001 (SEQID#1) and S98-002 (SEQID#2) both selectively replicate in cancer cells where the tumor-suppressing gene *p53* is dysfunctional.

[0086] ***In vitro* plaque forming test.** The *in vitro* plaque forming test was used to determine the growing ability of S98 viruses in p53 deficient cells. The cell lines used in this series of tests were: OVCAR-3 (oophoroma cell line, p53 deficient), Hep3B (hepatoma cell line, p53 deficient), U373 (glioma cell line, p53 deficient), SW620 (colon cancer cell line, p53 deficient), RKO (colon cancer cell line, wild type p53), HBL-100 (normal breast cell line, wild type p53).

[0087] S98-100 was used as the positive control to determine the oncolytic specificity for S98 viruses, as S98-100 replicate normally in cancer cells as well as in normal cells. Likewise, HYH was used as positive control for tested cell lines, as all of S98 viruses form plaques in HYH cells efficiently. To quantitatively compare the replication extents of S98 viruses, the plaques of S98-100 virus formed in HYH cell culture was arbitrarily defined as 100. The plaque number for other S98 viruses ("S98-XXX viruses") in any other type of cell line ("Z") was expressed as a percentage of the plaque numbers formed from a S98-XXX virus in cell line "Z" to the plaque number of S98-100 in HYH cells. This percentage is expressed as:

$$\left(\frac{\text{(Plaque Number of a S98-XXX virus in cell line - "Z")}}{\text{(Plaque Number of a S98-100 virus in cell line - "HYH")}} \right) \times 100 = \text{Percentage of Plaque Number in cell line "Z"}$$

[0088] Thus, by definition, a larger number of viral plaques represents faster virus replication. Table 1 shows that selective replication of a genetically engineered S98 adenoviruses in human cancer cells with p53 deficiency can be measured by plaques forming tests. For example, shown in Table 1, S98-001 (SEQID#1) and S98-002 (SEQID#2) replicate predominantly faster in cell lines with *p*53 deficiency than in cell lines without *p*53 deficiency. For example, S98-001 (SEQID#1) and S98-002 (SEQID#2) replicate much more rapidly in OVCAR-3 (oophoroma cell line, p53 deficient), Hep3B (hepatoma cell line, p53 deficient), U373 (glioma cell line, p53 deficient), and SW620 (colon cancer cell line, p53 deficient) cell lines when compared to the RKO (colon cancer cell line, wild type p53) and HBL-100 (normal breast cell line, wild type p53) cell lines. Although not wanting to be bound by theory, the plaques formed in cells with normal p53 are very much limited for S98-001 (SEQID#1) and S98-002 (SEQID#2) comparing to S98-100. For example, the plaque numbers of S98-001 (SEQID#1) and S98-002 (SEQID#2) are only respectively 1/470 and 1/250 of that of S98-100 in RKO cells (colon cancer cell line, wild type p53). Similarly, the plaque numbers of S98-001 (SEQID#1) and S98-002 (SEQID#2) are only respectively 1/3000 and 1/1000 of that of S98-100 in HBL-100 cells (normal breast cell line, wild type p53).

Table 1

Cell Line / Virus	p53 deficient cell lines					p53 wt cell lines	
	HYH	OVCAR-3	Hep3B	U373	SW620	RKO	HBL-100
S98-100	100	100	100	100	100	100	100
S98-001 (SeqID#1)	100	25	62	13	42	0.21	0.03
S98-002 (SeqID#2)	100	52	71	29	19	0.40	0.08

[0100] The results of these plaque forming tests exhibit that (1) S98-001 (SEQID#1) and S98-002 (SEQID#2) replicate selectively in cancer cells with *p*53 deficiency; (2) in cells with functional *p*53, the replication rate of S98-001 (SEQID#1) and S98-002 (SEQID#2) is extremely low, in contrast to S98-100 that has the similar replication rate to the wild-type adenoviruses.

[0101] ***In vitro* toxicity test.** The purpose of this series of tests is to determine the toxicity of S98-001 (SEQID#1) and S98-002 (SEQID#2) to normal cells. Human mircovessel endothelium cell (hMVEC) was chosen for these tests. hMVEC is originated from human lung tissue, and it is a kind of primary cell that does not regenerate. Cells were infected with S98 viruses in gradually increasing multiplicity of infection ("MOI"), and the pathological status of the cell cultures were continuously tracked. It was demonstrated that wild-type adenoviruses lyse the monolayers of cultured hMVEC's completely in 10 days after viral infection at the MOI of 0.01. In contrast, no pathological change was observed as late as on the 10^{th} day after infection with S98-001 (SEQID#1) or S98-002 (SEQID#2) at the MOI's of 0.01, 0.1, 1.0, and 10. Thus, the toxicity of S98-001 (SEQID#1) and S98-002 (SEQID#2) to normal cells was far less than wild-type adenoviruses.

EXAMPLE 2

[0102] In order to determine an effective dosing protocol for animals (e.g. including humans) to be treated with the composition and method of this invention, 5 dose levels were utilized for H101 (SEQID#1). The recombinant adenovirus was administered via intratumoral injection to patients having advanced solid tumors. One objective was to

determine the Maximal Tolerated Dose ("MTD") and safety of a intratumoral injection of H101 (SEQID#1). The five levels of H101 (SEQID#1) that were utilized are shown in Table 2 and a dosage escalation curve is shown in Figure 3. Three patients for each of the 5 separate dose levels were included. The MTD was determined to be the dose at which two patients experienced a DLT, wherein a DLT comprises a grade 4 toxicity for flu-like symptoms due to H101 (SEQID#1), a grade 4 toxicity for local reaction at the H101 (SEQID#1) injection site, or any other toxicity of grade 3 severity due to H101 (SEQID#1). If one of the three patients had a DLT, a total of 6 patients would be treated for that cohort.

Table 2.

Level	H101 (virus particle)
1	5.0×10^{7}
2	5.0×10^{9}
3	5.0×10^{10}
4	5.0×10^{11}
5	1.5×10^{12}

[0103] Figure 4 shows that 15 patients were enrolled with various types of tumors. Efficacy evaluation tumor assessment was performed only at the tumors injected with H101 (SEQID#1) because it is a product for local injection having 1 Partial Response ("PR") at level of $1.5x10^{12}$ (viral particles); 1 Minimal Response ("MR") at level of $5.0x10^{11}$ (viral particles) using non-conventional measurements.

[0104] The immune reaction after administration of H101 (SEQID#1) and the environmental impact contamination of excreted H101 was also determined. All samples taken after the administration of H101, including swabs of oropharynx, urine were negative. Plasma sample taken 4 days later after H101 administration were negative. Although not wanting to be bound by theory, these data suggest that H101 (SEQID#1) did not persist in the circulation or in the urine.

EXAMPLE 3

[0105] Treatment of cancer patients using oncolytic virus S98-001 (SEQID#1) synchronized with hyperthermia. The patient's date of birth was June 10, 1943. He was diagnosed "nasopharyngeal carcinoma" in 1990. After a period of treatment with radiotherapy, the progression of the primary tumor was controlled clinically. However, two tumors in the region of right neck and upper clavicle were progressing slowly during these years. In late 2001, these two tumors were treated by radiotherapy (Cobalt-60, DT 34 Gy/17F/24d) in combination with hyperthermia. Unfortunately, the progression of the two tumors was not suppressed by these treatments. This patient was hospitalized early in February 2002, and a physical examination for this patient was conducted before being treated by administration of oncolytic virus S98-001 (SEQID#1) synchronized with hyperthermia. This patient's general physical status was good, though his nasopharyngeal tissue was thickened tuberculously and engorged slightly. The surfaces of the two tumors were rough, thickened and hardened. The two tumors had the dimensions of $47\times26\times22$ mm^3 and $33\times25\times6$ mm^3 , and denoted No.1 tumor and No. 2 tumor respectively. The laboratory tests were carried out for this patient and the results of these tests are as follows: Blood Rt – normal; Urine Rt – normal; Dejection Rt – normal; Function of liver and kidney - normal, except GLO 24.2, ALT 45; Cell Immunology - normal, except CD3 60, CD4 39; X ray of chest - normal; Ultrasound - normal, except slight enlargement of spleen; ECG - complete block of right bundle; CT - two large tumors at right neck and upper clavicle, borderlines not clear.

[0106] The patient was diagnosed as: advanced nasopharyngeal carcinoma with metastasis on right shoulder and right neck. With the patients consent, he was treated by intratumoral administration of S98-001 (SEQID#1) synchronized with hyperthermia. In the course of treatment, the No. 1 tumor of the patient was injected intratumorally with S98-001 (SEQID#1) at 1.0×10^{12} viral particles for 5 consecutive days starting from the first day of the course. In contrast, the No. 2 tumor was not administrated with S98-001 (SEQID#1). The No. 1 tumor was then heated locally at 41-44°C for 90 min for 13 consecutive days starting from the 2^{nd} day of the course. A spectrum generator with the wave length at 4-24 um and penetrability at 4-5 mm was used for hyperthermia. While heating the No. 1 tumor, the No. 2 tumor was shielded to insure no hyperthermic treatment applied to this tumor. On the 22^{nd} day of the course, this patient's physical status was re-examined. It was found that, though the treatment including injection of S98-001 (SEQID#1) and local hyperthermia was only

applied to No. 1 tumor, both the No. 1 tumor (the treated tumor) and the No. 2 tumor (the not-treated tumor) had regressed visibly. Further measurements revealed that the size of the No. 1 tumor regressed from 47×26×22 mm^3 to 44×18×10 mm^3 (a 70.5% reduction). More significantly, the No. 2 tumor (a not-treated tumor) also regressed from 33×25×6 mm^3 to 23×17×5 mm^3 (a 52.6% reduction).

[0107] Although not wanting to be bound by theory, the advantages of this invention are summarized as following: (1) complete exposure of patient's CRA's to HSP's induced by hyperthermia, and subsequent presentation of the complete set of CRA's to immune system mediated by HSP's and DCs upon cancer cell lysis by oncolytic viruses; (2) synchronous expression of HSP's and lysis of cancer cells by oncolytic viruses insuring enough signals of CRA's presented to immune system in order to elicit the immune response against cancer; (3) an entirely *in vivo* process bypassing the tedious procedures of the two technologies of individualized vaccination discussed previously; (4) a single agent (an oncolytic virus) in synchronization hyperthermia to elicit immunity against the complete set of CRA's of an individual tumor for every cancer patient; (5) this immunological therapy is effective for primary as well as metastatic cancers.

[0108] This case demonstrates that oncolysis in synchronization with hyperthermia is effective for a treated-tumor where the treatment is applied directly. In addition, the method is also effective for distal-tumors where a first tumor had been "treated," but neither injection of S98-001 (SEQID#1) nor hyperthermia had been applied to the not-treated or distal-tumors.

EXAMPLE 4

[0109] **Chondrosarcoma.** The female patient was born in 1982. In April of 2001, a tumor was found on her left lumbar and after surgery she was diagnosed as "soft tissue sarcoma." In October 2001, the tumor relapsed and increased in size. In February 2002, the size of the tumor was 21cm x 35cm as determined by physical examination. Pathology of a tumor biopsy showed that it was malignant tumor and a probable dediffenrentiation chondrosarcoma. A CT showed that the tumor dimensions were 15cm x 11cm with some eroded ribs nearby. Additionally, 2 metastatic lesions with dimension of 0.6 x 0.8cm on upper lobe of right lung were detected. After treatment with radiotherapy, a CT in March

2002 showed that tumor dimension was 13cm x 11cm, wherein the ribs nearby were eroded, and 2 metastatic lesions with dimension of 1.0 x 1.0cm on upper lobe of right lung were detected. In March 2002, the patient was treated with chemotherapy with regimen as IFO 2g d1~3 + E-ADM 40mg d1~3 + DTIC 200mg d1~5. The side effects were too severe for the patient to stand. With the patient's consent, she was treated by intratumoral administration of S98-001 synchronized with hyperthermia from July 2002. In the cycle of treatment, the tumor on left lumbar was injected intratumorally with S98-001 at 5.0×10^{11} viral particles for 5 consecutive days starting from the first day of the cycle. Using a radiofrequency hyperthermia system operating at a frequency in the range from about 5 MHz to about 15 MHz, the injected lesion was then heated locally at 41-44°C for 70 min for 7 consecutive days starting from the 6th day of the cycle. A CT in December 2002 showed that the size of the tumor was 8.0cm x 6.0cm (or a 66% deduction) wherein some ribs nearby were eroded. The 2 metastatic lesions with dimension of 1.0 x 1.0cm on upper lobe of right lung were detected. A CT in July, 2003 showed that 2 metastatic lesions on upper lobe of right lung disappeared. This case demonstrates that oncolysis in synchronization with hyperthermia is effective for a treated-tumor where the treatment is applied directly to a primary tumor. Additionally, this example demonstrates that the composition and methods of this invention are also effective for distal-tumors (metastasis), wherein the distal-tumor was not directly injected with S98-001 and did not have hyperthermia applied.

EXAMPLE 5

[0110] Non-small cell lung cancer. The male patient was born in 1933. He was diagnosed as "adenocarcinoma of right lung" after pathology test in December 2002. The phase was T3N1M1/IV having a KPS score of 60. A CT scan detected a tumor mass in the upper lobe of the lung having dimensions (3cm x 2cm), and a metastatic lesion in the lower lobe of left lung having dimensions (1cm x 1cm). With the patient's consent, he was treated by intratumoral administration of S98-001 synchronized with hyperthermia from January 2003. In a cycle of treatment, the tumor on right lung was injected intratumorally with S98-001 at 1.5×10^{12} viral particles on day 1 and day 8 of the cycle. Using a radiofrequency hyperthermia system operating at a frequency in the range from about 5 MHz to about 15 MHz, the injected lesion was then heated locally at 41-44°C for 2 consecutive days after the injection. After 2 cycles treatment, CT scan showed that the metastatic lesion in the lower

lobe of left lung disappeared, the injected lesion stayed stable. CT of the visit in October, 2003 showed that the metastatic lesion in the lower lobe of left lung disappeared showing a complete response ("CR"), wherein the objective response of the injected lesion having dimensions of (3cm x 1cm with a 50% reduction in size) was a partial response ("PR"). This case demonstrates that oncolysis in synchronization with hyperthermia is effective for a treated-tumor where the treatment is applied directly to the tumor. Additionally, the method is also effective for distal-tumors.

EXAMPLE 6

[0111] Colon cancer. The male patient was born in 1983. He was diagnosed as "cancer of colon (sigmoid), small intestine and pelvic cavity invasion, Duke's D and moderate differentiated adenocarcinoma," after radical surgery in April 2001. After surgery, from July 2001 to April 2002, he was treated with chemotherapy, wherein 5-FU, CDDP, MMC was used, together with levamisole, capecitabine, CPT-11, Taxus chinensis compound and Coix lachrymajobi oil. In October 2002, a metastatic lesion on his abdominal wall was found with the size of 3.5cm × 5.0cm, along with the symptoms of incomplete intestinal obstruction. With the patient's consent, he was treated by intratumoral administration of S98-001 synchronized with hyperthermia from November 12th to November 18th, 2002. In a cycle of treatment, the primary tumor was injected intratumorally with S98-001 at 1.5×10^{12} viral particles. The injected lesion was then heated locally at 41-44°C for 70 min for 2 consecutive days after the injection. From November 21th, 2002, low dosage chemotherapy was used with the regimen 5-FU 0.3 24h d1~5 + DDP 5mg d1~5 + CPT-11 0.1 d1,8 for 4 cycles. There were 3 weeks included in one cycle. A CT on 10/28/2002 showed an abdominal wall lesion having dimensions 3.5 × 5.0cm, rectal region tumor 1.2cm × 1.0cm (before treatment). On 12/30/2002 showed the abdominal wall lesion had been reduced to 3.7cm × 2.0cm and the rectal region tumor having dimensions 1.2 × 1.0cm. A CT on 02/11/2003 showed : abdominal wall lesion had been reduced to 2cm × 2.5cm and the rectal region tumor had been reduced to 1.2cm × 1.4cm. CT's on 01/20/2003 and a fine needle biopsy of the areas on 02/21/2003 showed that both the abdominal wall lesion and the rectal region tumor were only proliferation of granulation tissue and no cancer cells were found. Symptoms of the patient were relieved and he went on normal diet. This case demonstrates that oncolysis in synchronization with hyperthermia is effective for a treated-tumor where the treatment is applied directly. In addition, the method is also effective for distal-tumors.

[0112] Although not wanting to be bound by theory, the advantages of the compositions and methods of this invention are summarized as following: (1) complete exposure of patient's CRA's to HSP's induced by hyperthermia, and subsequent presentation of the complete set of CRA's to immune system mediated by HSP's and DCs upon cancer cell lysis by oncolytic viruses; (2) synchronous expression of HSP's and lysis of cancer cells by oncolytic viruses insuring enough signals of CRA's presented to immune system in order to elicit the immune response against cancer; (3) an entirely *in vivo* process bypassing the tedious procedures of the two technologies of individualized vaccination discussed previously; (4) a single agent (an oncolytic virus) in synchronization hyperthermia to elicit immunity against the complete set of CRA's of an individual tumor for every cancer patient; (5) this immunological therapy is effective for primary as well as metastatic cancers.

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